[1] Isolation of Human Platelets from Plasma by Centrifugation and Washing

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Blood platelets are readily activated by a number of stimuli to change their shape, extend pseudopods, aggregate, release the contents of their storage granules, form thromboxanes and prostaglandins, and provide sites on their membranes which take part in two steps of the intrinsic coagulation pathway.[1,2] An ideal isolation method should avoid activation of platelets, or any of these consequences of activation, and provide a suspension of platelets that responds to stimulation in the same was as do platelets in the circulation. In addition, it is desirable to have the platelets maintain this responsiveness for several hours to permit *in vitro* experiments.[2-4]

Although it might appear that plasma would be the ideal suspending medium, the anticoagulants that must be used have major effects on the response of platelets. Many preparations of heparin cause some aggregation of the platelets[5,6]; hirudin is expensive and does not block activated coagulation factors other than thrombin, e.g., factor Xa; and EDTA, because of its strong chelation of divalent cations, has a deleterious effect on membrane glycoproteins participating in platelet aggregation. Many studies have been done with citrated platelet-rich plasma. The low concentration of ionized calcium in this medium introduces a major artifact because when human platelets are brought into close contact with each other in such a medium (for example by ADP) the arachidonate pathway is activated, resulting in thromboxane formation and the release of granule

[1] M. A. Packham and J. F. Mustard, *in* "Blood Platelet Function and Medicinal Chemistry" (A. Lasslo, ed.), p. 61. Elsevier, New York, 1984.

[2] R. L. Kinlough-Rathbone, M. A. Packham, and J. F. Mustard, *in* "Methods in Hematology: Measurements of Platelet Function" (L. A. Harker and T. S. Zimmerman, eds.), p. 64. Churchill-Livingstone, Edinburgh, Scotland, 1983.

[3] J. F. Mustard, D. W. Perry, N. G. Ardlie, and M. A. Packham, *Br. J. Haematol.* **22**, 193 (1972).

[4] R. L. Kinlough-Rathbone, J. F. Mustard, M. A. Packham, D. W. Perry, H.-J. Reimers, and J.-P. Cazenave, *Thromb. Haemostasis* **37**, 291 (1977).

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[6] M. B. Zucker, *Thromb. Diath. Haemorrh.* **33**, 63 (1975).

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contents.[7-9] Human platelets in a medium with approximately physiological concentrations of ionized calcium do not behave in this way; although they aggregate in response to ADP, the arachidonate pathway is not activated and granule contents are not released. A major advantage of studying platelet reactions in artificial media instead of in citrated plasma is that physiological concentrations of ionized calcium can be used.

Like other cells, platelets maintain their functions best in media that are similar to tissue culture media. Protective protein (albumin is most commonly used) prevents them from adhering to the sides of containers and lessen the chance of activation during isolation and handling.[3,4,10,11] A source of metabolic energy (glucose) is essential,[12,13] and the pH should be controlled at the pH of plasma (7.35).[14,15] Buffer such as Tris [tris(hydroxymethyl)aminomethane] should be avoided[16] because, like other amines,[17] it inhibits some platelet responses and potentiates others.

Another problem is the development of refractoriness of platelets to ADP-induced aggregation[18-20]; when platelets have been exposed to ADP, they aggregate to a lesser extent (or not at all) when more ADP is added with stirring. Since platelets contain ADP in their amine storage granules, release of small amounts of ADP during the isolation of platelets, and during storage, may make the platelets unresponsive to ADP. To prevent this, enzyme systems that remove ADP should be included in platelet-suspending media.[2-4] (It should be pointed out that plasma itself contains

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[12] R. L. Kinlough-Rathbone, M. A. Packham, and J. F. Mustard, *J. Lab. Clin. Med.* **75,** 780 (1970).

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[16] M. A. Packham, M. A. Guccione, M. Nina, R. L. Kinlough-Rathbone, and J. F. Mustard, *Thromb. Haemostasis* **51,** 140 (1984).

[17] R. L. Kinlough-Rathbone, M. A. Packham, and J. F. Mustard, *Thromb. Haemostasis* **52,** 75 (1984).

[18] J. R. O'Brien, *Nature (London)* **212,** 1057 (1966).

[19] S. Holme, J. J. Sixma, J. Wester, and H. Holmsen, *Scand. J. Haematol.* **18,** 267 (1977).

[20] T. J. Hallam, P. A. Ruggles, M. C. Scrutton, and R. B. Wallis, *Thromb. Haemostasis* **47,** 278 (1982).

enzymes that dephosphorylate ADP.[21]) The most commonly added enzyme is apyrase (EC 3.6.1.5), prepared from potatoes,[22] although creatine phosphate/creatine phosphokinase (EC 2.7.3.2) has also been used. However, platelets that are refractory to ADP-induced aggregation can be aggregated by other aggregating and release-inducing agents. It is almost impossible to prepare a platelet suspension that is unresponsive to strong agonists such as thrombin.

Methods

Collection of Blood

Because so many drugs affect platelet reactions (particularly aspirin and other nonsteroidal antiinflammatory drugs) donors should be carefully questioned about the drugs they have taken during the previous 2 weeks. The donors should not be stressed and it is preferable that blood be collected in the morning so that the donors are fasting and have not smoked for a number of hours.

Collect blood from a forearm vein with or without venous occlusion. If venous occlusion is used, the sphygmomanometer cuff should not be inflated above 60 mmHg (8 kPa). Care should be taken to avoid prolonged stasis and anoxia. A 19-gauge needle, or larger, should be used. Blood should be mixed immediately with the anticoagulant and frothing should be avoided during the mixing procedure. The first 1 – 2 ml of blood may be discarded to avoid the effects of traces of thrombin that have been shown to be generated during venepuncture.

Reagents

Plasticware and Glassware. Containers may be polyethylene, polycarbonate, or siliconized glass. Use a silicone preparation that binds firmly to the glass surface so that small micelles of silicone do not contaminate the platelet preparation. A suitable preparation is Surfasil (dichlorooctamethyltetrasiloxane, Pierce Chemical Co., Rockford, IL) prepared as a 10% solution in carbon tetrachloride or tetrahydrofuran. After coating, the glass surfaces should be thoroughly rinsed and dried.

Acid-Citrate-Dextrose (ACD). The acid-citrate-dextrose anticoagulant solution of Aster and Jandl[23] has the advantage of not only chelating

[21] I. Holmsen and H. Holmsen, *Thromb. Diath. Haemorrh.* **26,** 177 (1971).
[22] J. Molnar and L. Lorand, *Arch. Biochem. Biophys.* **93,** 353 (1961).
[23] R. H. Aster and J. H. Jandl, *J. Clin. Invest.* **43,** 843 (1964).

the calcium in the blood and thus preventing the activation of coagulation, but also of lowering the pH of the blood to 6.5; platelets do not aggregate readily at this pH. Prepare this anticoagulant by dissolving 25 g of trisodium citrate dihydrate, 15 g of citric acid monohydrate, and 20 g of dextrose in 1 liter of distilled water. This solution has an osmolarity of 450 mOsm/liter and a pH of about 4.5. One part of this anticoagulant is required for each six parts of blood.

Stock Solutions. Stock solutions for preparing Tyrode's buffer used for the preparation of washed platelets (may be stored 2 weeks at 4°) are as follow:

Stock I: This consists of NaCl (160 g), KCl (4.0 g), $NaHCO_3$ (20 g), and NaH_2PO_4 (1.0 g) made up to 1 liter with distilled water

Stock II: $MgCl_2 \cdot 6H_2O$ (20.33 g) made up to 1 liter to produce a 0.1 *M* solution

Stock III: $CaCl_2 \cdot 6H_2O$ (21.91 g) made up to 1 liter to produce a 0.1 *M* solution

Tyrode's Solution. Tyrode's solution containing albumin and apyrase (washing and resuspending fluid) is made up as follows: Add 750 ml of distilled water to 50 ml of stock I, add 10 ml of stock II (with mixing), and 20 ml of stock III (with mixing). Dissolve 3.5 g Pentex fraction V bovine serum albumin (Miles Laboratories, Kankakee, IL) and 1 g of dextrose in this solution and bring to a total volume of 1 liter with distilled water. Adjust the pH to 7.35 and the osmolarity to 290 mOsm/liter by addition of NaCl if necessary. Human serum albumin may be used in place of bovine albumin. Add apyrase in the concentrations indicated below.

Optional Final Resuspending Fluid. Eagle's medium (Cat. No. 410-1100, Gibco, Grand Island, NY) is supplemented with the following additions: 2.2 g/liter $NaHCO_3$, 0.35% albumin, and apyrase.

Apyrase. Apyrase can be prepared by a modification of the method of Molnar and Lorand[2,22] in a two-stage procedure:

Stage I is conducted at room temperature. Wash and slice potatoes (10 kg) and homogenize in a Waring blender with 1 liter of distilled water. Stir the homogenate for 30 min and then centrifuge it at 900 *g* for 10 min. Filter the supernatant fluid through multiple layers of cheesecloth and measure the volume of the effluent.

Stage II is conducted at 0–4°. Add $CaCl_2$ to the supernatant in sufficient quantity to bring the solution to 0.025 *M* $CaCl_2$, stir for 15 min, allow the mixture to settle for 1 hr, and centrifuge for 20 min at 3500 *g*. Resuspend the precipitate in 1 *M* $CaCl_2$ to a volume that is approximately one-tenth that of the original effluent and stir for 60 min before centrifuging for 20 min at 3500 *g*. Dialyze the supernatant against 0.1 *M* KCl (use

20 liters for material from 10 kg of potatoes) for 24 hr and then centrifuge for 20 min at 3500 *g*. Fractionate the supernatant fluid by adding 3 vol of ice-cold saturated ammonium sulfate solution to each volume of supernatant fluid. Stir for 40 min and then centrifuge for 15 min at 3500 *g*. Dissolve the precipitate in a minimum volume of distilled water and dialyze against several changes of 0.154 *M* (0.9%) NaCl.

Finally, assay the protein by the Folin–Ciocalteau method[24] and dilute to a concentration of 3 mg/ml. Store the enzyme in small aliquots at −20°. The nucleotidase activity of the apyrase preparation can be determined by measuring the rate at which it degrades $[^{14}C]$ATP to $[^{14}C]$ADP and $[^{14}C]$AMP.[2,25] Some commercially available preparations are not suitable because they contain impurities and 5′-nucleotidase activity that hydrolyzes $[^{14}C]$AMP to $[^{14}C]$adenosine. Use a concentration of apyrase in the final suspending medium that is capable of converting 0.25 μM ATP to AMP in 120 sec at 37°. Alternatively, choose an apyrase concentration that maintains platelet sensitivity to ADP, but does not have an appreciable inhibitory effect on ADP-induced aggregation (tested in the presence of fibrinogen). If too much apyrase is used, ADP-induced aggregation will be inhibited and if too little is used, the platelets will become refractory to ADP.

Fibrinogen. Fibrinogen can be partially purified by the method of Lawrie *et al.*[26] to remove contaminating factor XIII, plasminogen, and fibronectin.

Heparin. Some preparations of heparin cause platelet aggregation[5,6] and cause platelets to stick to the walls of their container. Batches of heparin should be screened before use to ensure that this does not occur.[2]

Preparation of Suspensions of Washed Human Platelets

The procedure can be used to prepare platelets from 120–150 ml of blood, but can be adapted for larger volumes provided the proportions of washing and resuspending solutions are maintained. The platelets are kept at 37° throughout this procedure.

Collect blood into ACD anticoagulant (6/1, v/v) in 50-ml polycarbonate tubes. Centrifuge at 190 *g* for 15 min at 37° to obtain platelet-rich plasma and transfer the plasma to fresh, 50-ml conical polycarbonate tubes with a siliconized Pasteur pipet or a plastic syringe. (Note: Occasionally a lower *g* force may be required, depending on the characteristics of the

[24] J. M. Clark, Jr., and R. L. Switzer, "Experimental Biochemistry," 2nd Ed. Freeman, San Francisco, California, 1977.

[25] M. A. Packham, N. G. Ardlie, and J. F. Mustard, *Am. J. Physiol.* **217**, 1009 (1969).

[26] J. S. Lawrie, J. Ross, and G. D. Kemp, *Biochem. Soc. Trans.* **7**, 693 (1979).

platelets, plasma, and red cells of individual donors.) Centrifuge the platelet-rich plasma at 2500 *g* for 15 min at 37°, discard as much of the plasma as possible, and gently resuspend the platelets, using a siliconized Pasteur pipet, in 10 ml of Tyrode's solution containing heparin (50 U/ml), albumin (0.35%), and apyrase (use approximately 10 times the amount of apyrase that is required to stabilize platelets in suspending medium), pH 7.35, 37°. It is *essential* that platelets be left in this solution at 37° for at least 15 min. Centrifuge at 1200 *g* for 10 min at 37°, discard the supernatant fluid, and gently resuspend the platelets in Tyrode's solution containing albumin (0.35%) and the same amount of apyrase as used in the first washing solution. If there are a few red cells at the bottom of the tube, avoid resuspending them with the platelets. Centrifuge once more (1200 *g* for 15 min), discard the supernatant fluid, and gently resuspend the platelets in Tyrode's solution containing albumin (0.35%) and apyrase.

Platelets prepared using this method and stored in a water bath at 37° remain responsive to ADP in the presence of fibrinogen (75–200 μg/ml) for 3–4 hr.[4] If even longer periods of storage are to be used, HEPES buffer (5 m*M*, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, Sigma) may be added to the suspending medium.[27] Platelets in these media are also responsive to all other aggregating and release-inducing agents such as collagen, thrombin, sodium arachidonate, the divalent cation ionophore A23187, and platelet-activating factor among others. Additional fibrinogen is not required when a release-inducing agent is used since fibrinogen released from the platelet α granules in response to release-inducing agonists is sufficient to support platelet aggregation. In this calcium-containing medium, platelets do not respond consistently to epinephrine,[2] even in the presence of fibrinogen, unless traces of another agonist are present.[28]

Comments

Potential Problems and Pitfalls

Problems are apt to arise when the procedure is shortened or necessary reagents are omitted. Maintaining the temperature at 37° ensures that the platelets are not activated by cooling and rewarming. The times of incubation in the washing procedure must not be shortened below 15 min; they may be longer without deleterious effects. It is essential that a protective protein be present. Albumin is most satisfactory and may be used at

[27] S. Timmons and J. Hawiger, *Thromb. Res.* **12,** 297 (1978).

[28] J. F. Mustard, C. Lalau-Keraly, D. W. Perry, and R. L. Kinlough-Rathbone, *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **44,** 1127 (1985).

concentrations between 0.35% and 4%. The latter concentration is similar to that in plasma, but investigators should be aware of the ability of albumin to bind drugs, fatty acids, etc. For example, the concentration of arachidonic acid required to stimulate platelets in 4% albumin is much greater than that required in 0.35% albumin. If albumin is omitted entirely, some activation occurs when the platelets are merely stirred; prelabeled platelets release 5 to 10% of their [^{14}C]serotonin and lose 2% of their ^{51}Cr upon stirring in an aggregometer cuvette, whereas in the presence of 0.35% albumin only 0.1 to 0.4% of the [^{14}C]serotonin is released and only 0.5% of the ^{51}Cr.[4] In addition, without albumin in the suspending medium, the response to ADP (in the presence of fibrinogen) is variable.

Omission of apyrase or the creatine phosphate/creatine phosphokinase system, either of which removes any ADP that accumulates in the suspending medium, causes the platelets to become progressively more and more refractory to ADP, although they will continue to respond to other aggregating agents.

Omission of Ca^{2+} from the suspending medium introduces the same artifact that occurs in citrated platelet-rich plasma (Table I). When human platelets are brought into close contact in a medium without added calcium, the arachidonate pathway is activated, thromboxane A_2 forms, the platelets release some of their granule contents, and second phase aggregation occurs; aspirin and other nonsteroidal antiinflammatory drugs inhibit this secondary activation by blocking thromboxane A_2 formation. ADP causes only the primary phase of aggregation in a medium containing 1 – 2 mM Ca^{2+}, regardless of the concentration of ADP used. However, if traces of release-inducing stimuli are present, addition of ADP may lead to activation of the arachidonate pathway, even in the presence of 2 mM calcium.[3] The omission of Ca^{2+} from platelet-suspending media has led to many results of dubious significance in regard to physiological conditions.

Advantages of the Method

The advantages of this method are that large volumes of blood can be handled readily, the reagents required are inexpensive, the platelet count in the final suspension can be adjusted to any desired number, platelets respond to aggregating and release-inducing agents in a manner similar to platelets in native plasma or plasma anticoagulated with hirudin, and maintain this responsiveness for hours.[4] The constituents of the medium can be varied, or additions made, as required for experimental purposes. Thrombin can be used as an agonist without the problem of the formation

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TABLE I
EFFECT OF Ca^{2+} ON RELEASE OF [^{14}C]SEROTONIN[a]

Ca^{2+} in platelet-suspending medium	ASA (500 μM)	^{14}C in suspending fluid (% of total in platelets)
2 mM	−	0.3
2 mM	+	0.2
0	−	43.0
0	+	0.4

[a] Prelabeled human platelets stimulated with 2.5 μM ADP. Fibrinogen (200 μg/ml) and ADP were added to the platelet suspensions stirred at 1100 rpm in aggregometer cuvettes at 37°. The release of [^{14}C]serotonin from prelabeled platelets was measured after 3 min, as described elsewhere.[4] In the presence of 2 mM Ca^{2+}, only the primary phase of platelet aggregation occurred. When no Ca^{2+} was added to the medium, ADP induced two phases of aggregation accompanied by the release of platelet granule contents. This second phase was blocked by aspirin (ASA), added 5 min before ADP in this experiment.

of large amounts of fibrin that occurs when thrombin is added to platelet-rich plasma. The morphological appearance of the platelets by electron microscopy is similar to that of platelets in plasma.[4] Since the platelet suspensions are kept at 37°, experiments can be readily done at this temperature so that conditions more closely resemble the *in vivo* situation.

Although there are a number of other isolation procedures, most of them are less suitable for large volumes of blood and are more costly. Gel filtration[11,29] suffers from the problem that platelet counts are low because of dilution on the column and proteins of high molecular weight may be eluted with the platelets unless special precautions are taken.[27] Addition of PGI_2 to prevent activation of platelets during isolation appears to be satisfactory,[30] but is prohibitively expensive for routine use in most laboratories. Separation on density gradients of albumin,[31] Stractan,[32] or sodium metrizoate[33] is not suitable for large-scale preparations.

The method of isolation described here has proved to be most satisfactory for many types of *in vitro* experiments involving aggregating agents, inhibitors, and various conditions that affect platelet responses. A major

[31] P. N. Walsh, D. C. B. Mills, and J. G. White, *Br. J. Haematol.* **36**, 281 (1977).
[32] L. Corash, B. Shafer, and M. Perlow, *Blood* **52**, 726 (1978).
[33] P. Ganguly, and W. J. Sonnichsen, *J. Clin. Pathol.* **26**, 635 (1973).

advantage is that the response of platelets to ADP (in the presence of added fibrinogen) remains similar to that in native plasma. Aggregation in response to ADP, without thromboxane production or release of granule contents, is the characteristic of a good preparation.

[2] Isolation of Human Platelets by Albumin Gradient and Gel Filtration

By SHEILA TIMMONS and JACEK HAWIGER

Human blood platelets interact with several plasma proteins that participate in the formation of a hemostatic plug.[1] To study the interactions of plasma proteins with their receptors on the platelet membrane a preparation of platelets free of plasma proteins is needed in order to assure that they will not interfere with the binding of labeled ligands. A number of methods provide platelets that have been used for testing the effect of plasma proteins and general requirements have been formulated.[2] The methods which employ repeated washing and centrifugation work best when a relatively large volume of blood is used.[3] Isolation of platelets from small samples of blood, i.e., 20–40 ml, by repeated washing and centrifugation usually ends with a poor yield of platelets. In some instances washed platelets are treated with formalin for agglutination studies.[4] Such treated platelets cannot be used for studying processes requiring metabolically intact platelets, e.g., signal transduction generated by ADP, epinephrine, and arachidonic acid.

The second group of methods of separation of platelets from plasma proteins includes gel filtration and/or centrifugation over a cushion or gradient made of albumin, Ficoll, or stractan.[5-12] The gel filtration method

[1] J. Hawiger, *in* "Hemostasis and Thrombosis: Basic Principles and Clinical Practice" (R. Colman, J. Hirsh, V. Marder, and E. Salzman, eds.), 2nd Ed., p. 162. Lippincott, Philadelphia, Pennsylvania, 1987.

[2] H. J. Day, H. Holmsen, and M. B. Zucker, *Thromb. Diath. Haemorrh.* **33,** 648 (1975).

[3] J. F. Mustard, D. W. Perry, N. H. Ardlie, and M. A. Packham, *Br. J. Haematol.* **22,** 193 (1972).

[4] D. E. Macfarlane, J. Stibble, E. P. Kirby, and M.B. Zucker, *Thromb. Diath. Haemorrh.* **34**, 306 (1975).

[5] S. Timmons and J. Hawiger, *Thromb. Res.* **12,** 298 (1978).

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